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(54) Title: HYALURONIC ACID DERIVATIVES

(57) Abstract: Derivatives between hyaluronic acid and at least one nitrogenated base, in particular at least one heterocyclic compound derived from purine and/or from pyrimidine and cosmetic and/or pharmaceutical compositions based on said derivatives.

WO 2004/013182 A1

## "HYALURONIC ACID DERIVATIVES"

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### SUBJECT OF THE INVENTION

5           The subject of the present invention are new derivatives of hyaluronic acid, a process for the preparation of said derivatives, their use in the cosmetic and/or pharmaceutical field, and the cosmetic and/or pharmaceutical compositions that comprise them.

### STATE OF THE ART

10           Hyaluronic acid is a mucopolysaccharide present practically in every part of the living organism and in particular in the dermis. Chemically, hyaluronic acid is made up of linear polymeric chains which have a molecular weight of hundreds of thousands to millions of dalton, in which disaccharide units consisting of *N*-acetylglucosamine and glucuronic acid, bonded together by glucoside bonds, are  
15           repeated. Hyaluronic acid possesses the capacity for bonding considerable amounts of water coming from the capillary network, necessary for maintaining in solution the catabolytes, the electrolytes and the gases that are diffused from the capillaries to the cells and vice versa, through the interstitial liquid. Hyaluronic acid thus performs a fundamental role in controlling the diffusion of nutritive substances, hormones,  
20           vitamins and inorganic salts of the connective tissue and in removing the metabolic detritus that may give rise to reactions of an inflammatory type. Thanks to the characteristics indicated above, hyaluronic acid is very much exploited in cosmetic preparations, for example for restoring proper hydration of the tissues and thus counteracting the processes of ageing of the tissues, in particular of the skin. There  
25           have been known for some time derivatives of hyaluronic acid obtained by means of formation of covalent bonds between hyaluronic acid and other compounds. For example, there are known amides of hyaluronic acid, to obtain which there is formed a covalent bond between the carboxyl group of hyaluronic acid and an amine group present on the reagent. These amide derivatives are prepared, for example, by means

of activation of the carboxyl group (for example, by transformation of the same in an activated ester) and subsequent reaction with the amine group of the reagent. The reaction envisages the formation of a new covalent bond and the elimination of a molecule of water (or, for example, of hydrochloric acid in the case where the ester is  
5 activated) for each amide bond that is formed. There are also known esters of hyaluronic acid, where the carboxyl group (possibly activated in an appropriate way) has reacted with an hydroxyl derivative to form a new bond of a covalent type (ester), with elimination, also in this case, of a molecule of water (or acid) for each  
10 bond of an ester type obtained. All these derivatives have in common the characteristic of proving extremely stable compounds and in particular resistant to enzymatic attack, so as to be able to guarantee, if administered, a greater resistance and duration of their effect over time.

#### OBJECTS OF THE INVENTION

An object of the present invention is to provide derivatives of hyaluronic acid  
15 that will be provided with bonds that are labile and readily hydrolysable in mild conditions, also by enzymatic effect.

A further object of the present invention is to make available derivatives of hyaluronic acid which are characterized by high efficacy in the cosmetic and pharmaceutical field and which have chemical-physical characteristics such as to  
20 enable use thereof in a wide range of types of formulations.

Yet another object of the present invention is to make available cosmetic and/or pharmaceutical compositions with a base of derivatives of hyaluronic acid, and a process for their preparation.

#### DESCRIPTION

25 The above and yet other purposes and corresponding advantages, which will be clarified in greater detail in what follows, are achieved by derivatives between hyaluronic acid and at least one nitrogenated base, in particular at least one heterocyclic compound derived from purine and/or from pyrimidine.

According to the invention, said hyaluronic acid is hyaluronic acid with high

molecular weight, said molecular weight being comprised in a range between 400 000 and 4 million dalton, preferably between 800 000 and 3.5 million dalton, and in particular between 1.5 and 3 million dalton.

Always according to the invention, said hyaluronic acid is hyaluronic acid  
5 with low molecular weight, said molecular weight being, for example, comprised in a range between 80 000 and 400 000 dalton.

According to the present invention, said derivatives of purine are chosen between adenine and/or guanine, while said derivatives of pyrimidine are chosen between thymine and cytosine. The aforesaid derivatives of purine and of pyrimidine  
10 are commonly referred to by the term "purine and pyrimidine bases". Once again according to the invention, other derivatives of purine and pyrimidine, commonly referred to by the term "minor bases", which may advantageously yield derivatives with hyaluronic acid, are the following:

5,6 dihydrouracyl  
15 1-methyluracyl  
3-methyluracyl  
5-hydroxymethyluracyl  
2-thiouracyl  
N<sup>4</sup>-acetylcytosine  
20 3-methylcytosine  
5-methylcytosine  
5-hydroxymethylcytosine  
1-methyladenine  
2-methyladenine  
25 7-methyladenine  
N<sup>6</sup>-methyladenine  
N<sup>6</sup>, N<sup>6</sup>-dimethyladenine  
N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine  
1-methylguanine

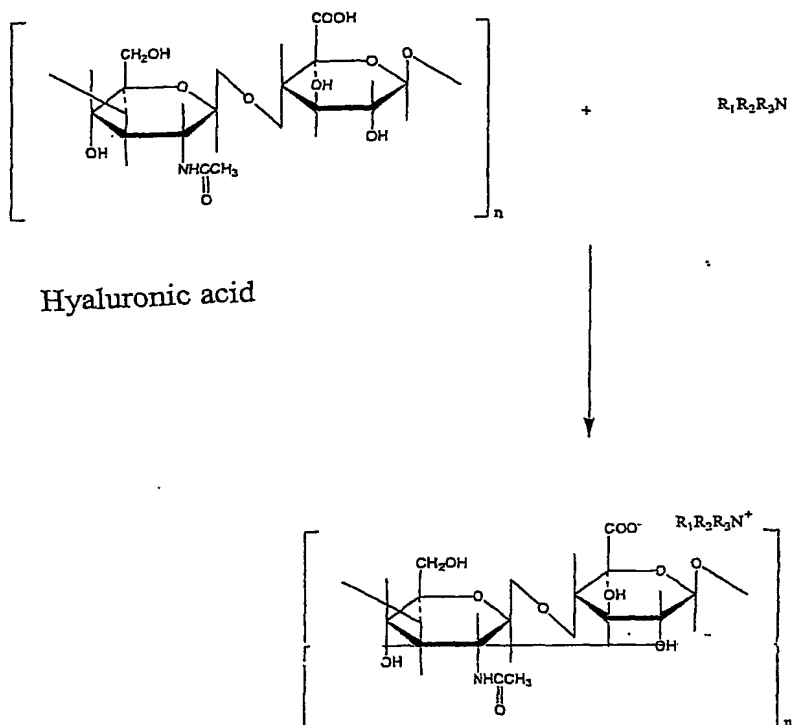
7-methylguanine

N<sup>2</sup>-methylguanine

N<sup>2</sup>, N<sup>2</sup>-dimethylguanine.

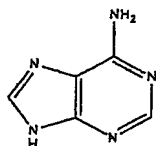
In practice, according to the present invention, hyaluronic acid is made to  
5 react with at least one purine and/or pyrimidine base chosen between the ones  
indicated above, in reaction conditions such as to form at least one bond of a ionic  
type between at least one "acid" centre of hyaluronic acid, such as for example a free  
carboxyl group in the form of an acid or in the form of a carboxylate salt, and at least  
one basic centre of the purine and/or pyrimidine base, which is also in the form of a  
10 free base or of an ammonium salt. The reaction scheme may in general be indicated  
as follows:

## SCHEME 1

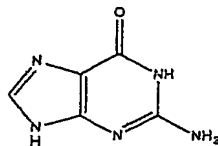


Where  $\text{R}_1\text{R}_2\text{R}_3\text{N}$  indicates generically at least one purine or pyrimidine base.  
 The bases most commonly used have the following formulae:

## PURINE BASES

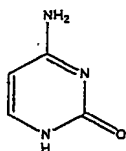


Adenine

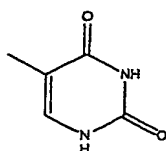


Guanine

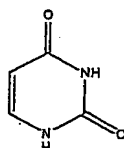
## PYRIMIDINE BASES



Cytosine



Thymine



Uracil

An important characteristic of purine and pyrimidine bases is their capacity of forming hydrogen bonds by means of the  $\text{-NH}_2$  groups where available, the  $\text{-NH-}$  groups and the markedly electronegative atoms of oxygen. At least one amine group characteristic of the bases referred to above, both just as they are and in the form of a salt, reacts with at least one carboxyl group of hyaluronic acid, (which is also just as it is in appropriate reaction conditions or in the form of a carboxylate salt) to yield a salt, characterized by the formation of at least one bond of a ionic type between the carboxyl group and the amine group. Always according to the present invention, by varying the reaction ratios between hyaluronic acid and the purine/pyrimidine bases, as well as by using bases different from one another for subsequent stages of salification, it is possible to prepare mixed salts between a number of purine/pyrimidine bases so as to obtain a "cocktail" with specific characteristics

suitable for the different applications.

Hyaluronic acid salified with at least one purine/pyrimidine base is characterized by a viscosity on average lower than that of the derivatives of hyaluronic acid according to the known art, characterized in that they are obtained by means of formation of covalent bonds. The derivatives of hyaluronic acid according to the present invention are thus characterized by a lower stability and duration in time as compared to those according to the known art, and this constitutes an advantage because they are able to make available hyaluronic acid just as it is, or other products or active principles linked thereto, rapidly after administration in that they undergo rapid hydrolysis of the ionic bond (or ionic bonds) that characterize them.

There appear hereinafter some examples of practical embodiment provided purely by way of indicative and non-limiting examples of the present invention.

EXAMPLE 1 - REACTION BETWEEN GUANINE AND HYALURONIC ACID to obtain GUANINE HYALURONATE (I)

1 g of sodium hyaluronate is made to react with 0.467 g of guanine hydrochlorate.

The preparation of the product takes place according to the following steps:

- a) preparation of sodium hyaluronate gel by mixing sodium hyaluronate with water;
- b) addition of guanine hydrochloride to the sodium hyaluronate gel;
- c) lyophilization of the product.

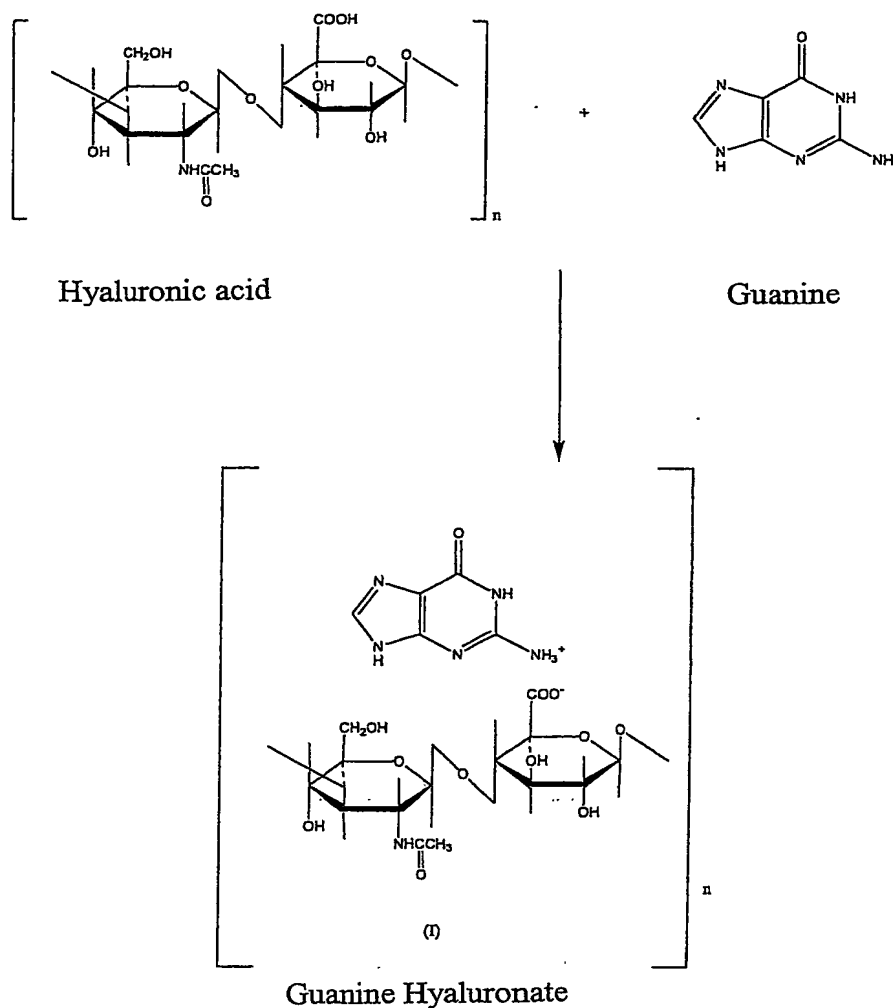
There are suspended under stirring 10 g of sodium hyaluronate in 300 ml of deionized water, up to complete gelification. The product appears clear, colourless, homogeneous and without clots.

The hyaluronic acid/water ratios are purely indicative; in the case where it is not intended to proceed to drying or lyophilization of the product and it is intended to use the gel directly, there may be used larger amounts of water (both at the start and at the end of the reaction) in order to obtain a product with more appropriate physical



characteristics (for example, viscosity). To the sodium hyaluronate gel previously prepared, there are then added slowly and under stirring 250 ml of solution containing 0.467 g of guanine hydrochloride. At the end of the addition, the product is kept under stirring for approximately 1 hour to obtain complete homogenization  
5 thereof. In the case where it is desired to modify the pH of the product, an acid or a base is added under the control of a pHmeter until the desired pH is reached.

The product obtained is kept in a refrigerator for 12 hours and then undergoes lyophilization or drying in vacuum conditions. A variation to the method described above consists in adding the hyaluronate gel to the solution of guanine  
10 hydrochloride.



To the product obtained according to the example referred to above, there may be associated other and different organic molecules that are co-ordinated thereto by means of hydrogen bonds.

5 EXAMPLE 2 - REACTION BETWEEN ADENINE AND HYALURONIC ACID to obtain ADENINE HYALURONATE (II).

1 g of sodium hyaluronate is made to react with 0.450 g of adenine hydrochloride hemihydrate.

The preparation of the product takes place according to the following steps:

10

- a) preparation of the gel by mixing sodium hyaluronate with water;
- b) addition of adenine hydrochloride hemihydrate to the sodium

hyaluronate gel;

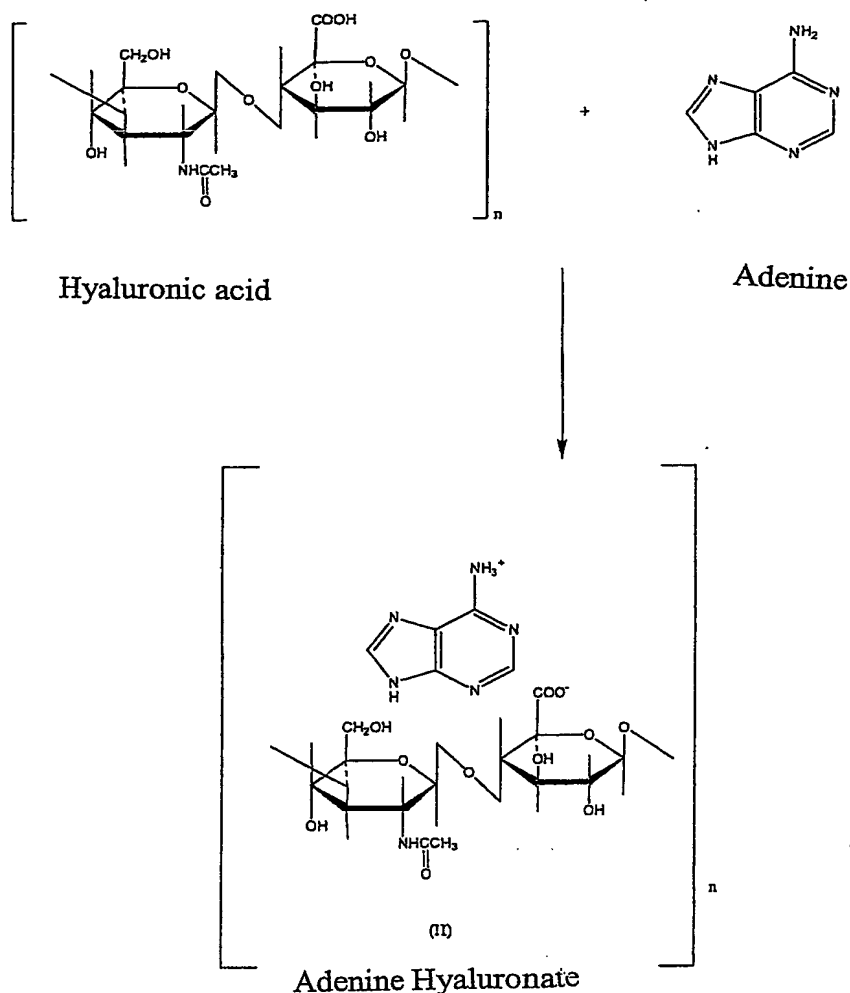
c) lyophilization of the product.

There are suspended, under stirring, 10 g of sodium hyaluronate in 300 ml of deionized water, up to complete gelification. The product appears clear, colourless,  
5 homogeneous and without clots. The hyaluronic acid/water ratios are only indicative. In the case where it is not intended to proceed to drying or lyophilization of the product, and it is intended to use the gel directly, it is possible to use larger amounts of water (both at the start and at the end of the reaction) in order to obtain a product with the most appropriate physical characteristics (for example viscosity).

10 To the sodium hyaluronate gel previously prepared, there are added slowly and under stirring 250 ml of solution containing 4.45 g of adenine hydrochlorate hemihydrate. At the end of the addition the product is kept under stirring for approximately 1 hour to obtain the complete homogenization thereof. In the case where it is desired to modify the pH of the product, an acid or a base is added under  
15 control of a pHmeter until the desired pH is reached. The product is left resting in a refrigerator for 12 hours, and this is followed by lyophilization or drying of the product in vacuum conditions.

A variation to the method indicated consists in adding the hyaluronate gel to the solution of adenine hydrochloride.

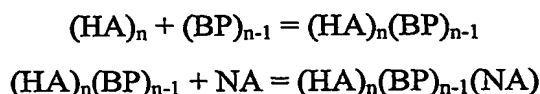
20



To the adenine hyaluronate derivative (II) obtained as described above it is possible to associate other and different organic molecules that are co-ordinated thereto by means of hydrogen bonds.

- 5 Natural amino acids, their oligomers and their polymers (peptides) can react with the derivatives of hyaluronic acid according to the invention (hyaluronic acid salified with at least one purine/pyrimidine base) to yield further products of salification with possible free carboxyl groups that are still present. In practice, if the aim is to schematize the reaction, and indicating the reagents as HA (hyaluronic acid), BP (purine/pyrimidine bases), and NA (natural amino acids, their oligomers and their polymers), we shall obtain the following scheme:
- 10

## SCHEME 2



For example, if guanine hyaluronate (I) or adenine hyaluronate (II) as described in the foregoing examples is obtained using a base deficiency with respect to hyaluronic acid, these can react in appropriate conditions with one or more natural amino acids and/or their oligomers and/or their polymers (peptides) to obtain further salts of hyaluronic acid that will be characterized, as well as by the presence of at least one purine/pyrimidine base, also by the presence of at least one natural amino acid or its oligomer or polymer. The characteristics of the final product will thus include both those of hyaluronic acid and those of the purine bases and of the amino acids, since also in this case the bond that is formed is of a ionic type with high ease of hydrolysis and hence the corresponding availability of the species just as they are.

EXAMPLE 3 - REACTION BETWEEN ADENINE HYALURONATE (II) AND POLYLYSINE

1 g of adenine hyaluronate (salified at 50% with adenine) is made to react with 0.143 g of polylysine. The preparation of the product takes place according to the following steps:

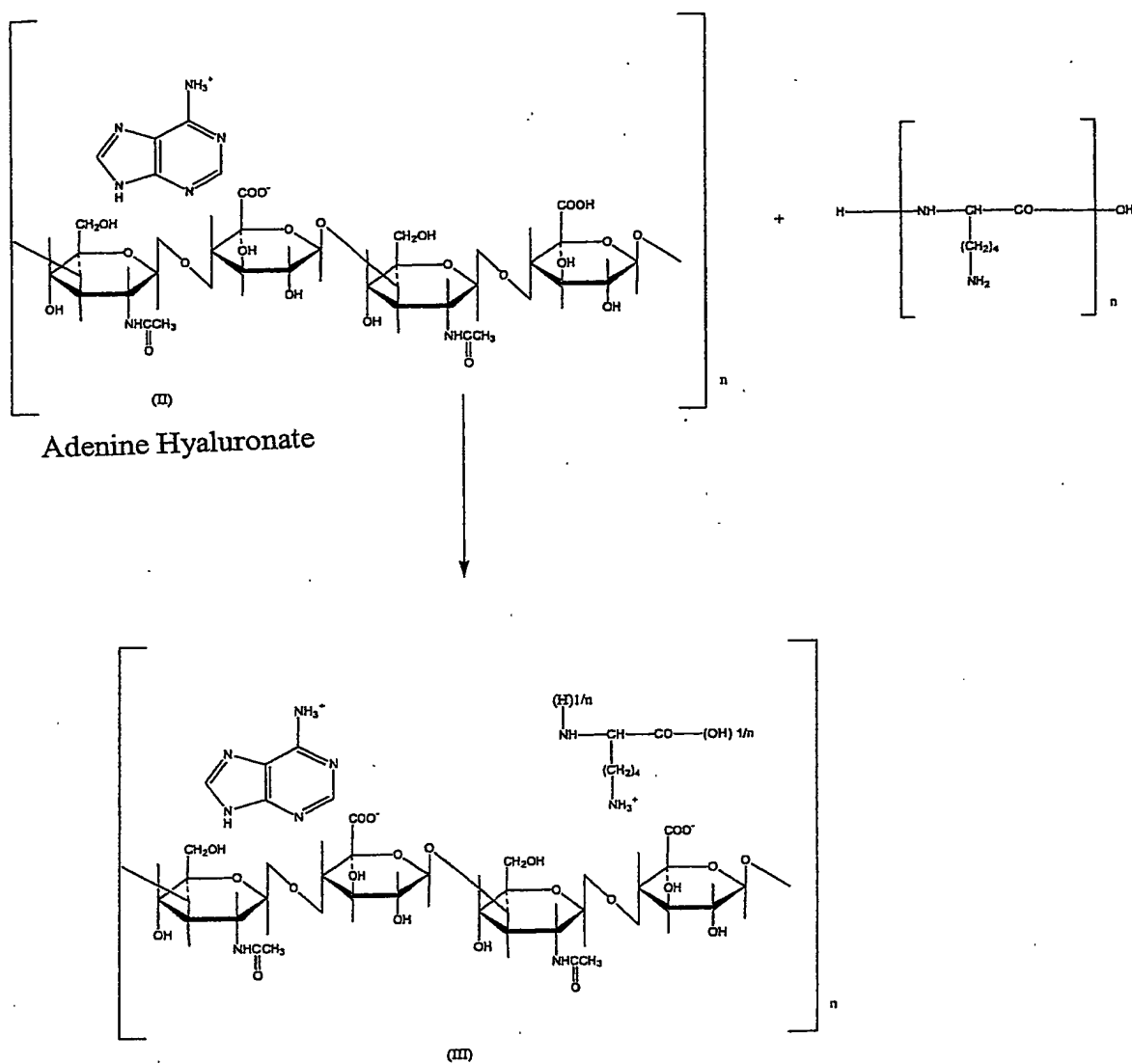
- a) preparation of the gel by mixing hyaluronic acid with water/Adenine;
- b) addition of the polylysine to the hyaluronic-acid gel/Adenine (compound II);
- c) lyophilization of the product.

There are suspended under stirring 10 g of hyaluronic acid in 300 ml of deionized water, up to complete gelification. The product appears clear, colourless, homogeneous and without clots. The gel obtained has a pH of 6.6.

The hyaluronic acid/water ratios are indicative. In the case where it is not intended to proceed to drying or lyophilization of the product and it is intended to use the gel directly, it is possible to use larger amounts of water (both at the start and at the end of the reaction) in order to obtain a product with the most appropriate

physical characteristics (for example viscosity). To the hyaluronic-acid/Adenine gel previously prepared (Compound II) there are added slowly and under stirring 200 ml of a solution containing 1.51 g of polylysine. At the end of the addition, the product is kept under stirring for approximately 1 hour to obtain complete homogenization thereof. In the case where it is desired to modify the pH of the product, an acid or a base is added under the control of a pHmeter until the desired pH is reached. The product is left resting in a refrigerator for 12 hours, and this is followed by lyophilization or drying of the product in vacuum conditions.

A variation to the method indicated consists in adding the hyaluronic-acid/Adenine (Compound II) to the solution of polylysine.



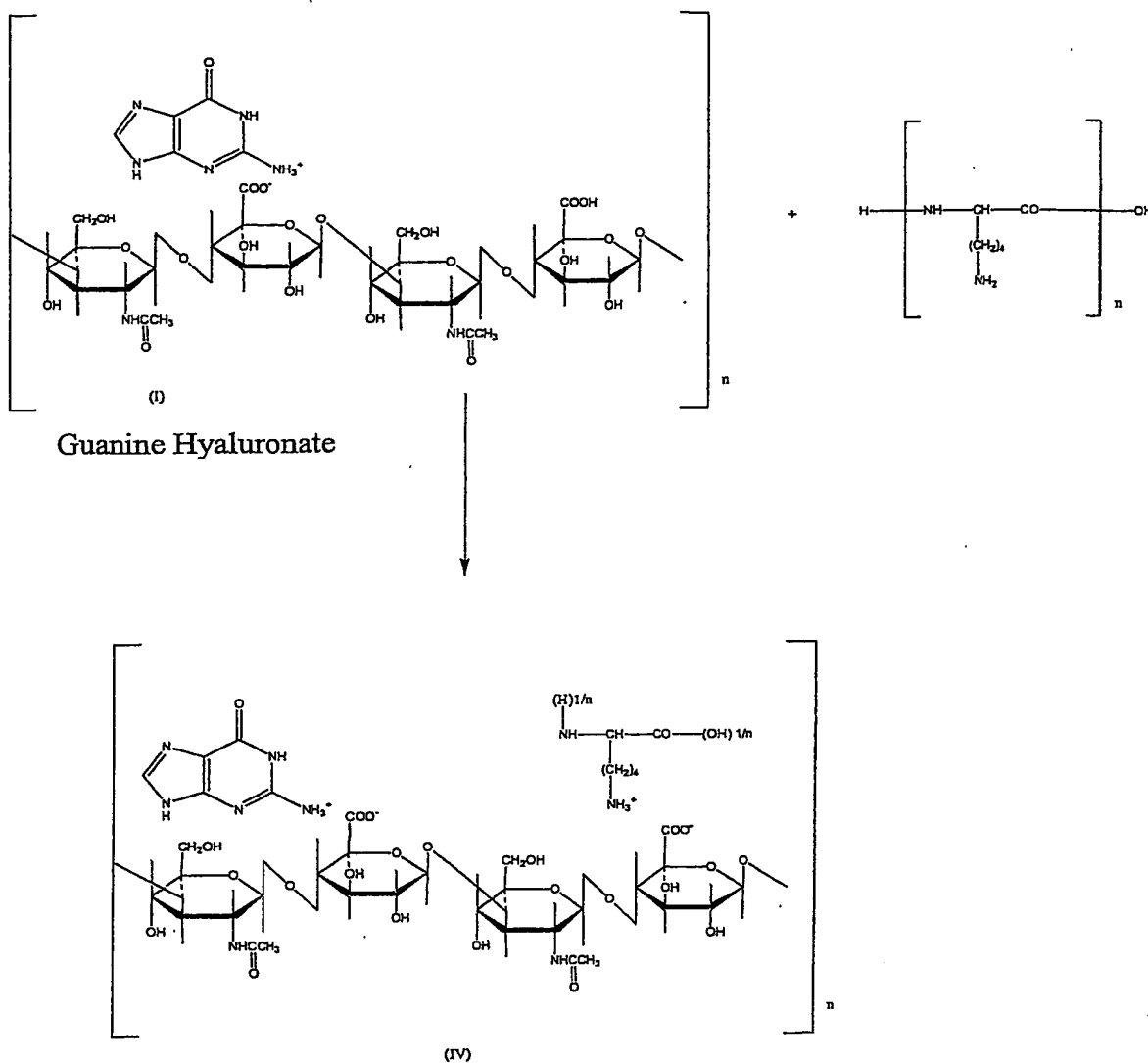
To the product obtained it is possible to associate other and different organic molecules that are co-ordinated thereto by means of hydrogen bonds.

As regards the product (III), as emerges from the scheme referred to above, it is understood that the unit deriving from the polylysine will be present  $n$  times according to the molar ratios between the reagents.

In the same way, the guanine hyaluronate (I) may react with polylysine or other oligomers or polymers (peptides) of amino acids.

EXAMPLE 4 - REACTION BETWEEN GUANINE HYALURONATE (I) AND

## POLYLYSINE



Also in this case, as regards the product (IV) as emerges from the scheme  
 5 appearing above, it is understood that the unit deriving from the polylysine will be present  $n$  times according to the molar ratios between the reagents.

The reactions of preparation of the derivatives forming the subject of the present invention may be conducted using, as has already been said, stoichiometric amounts of the reagents or an excess of one of the components. By stoichiometric



amounts of the reagents it is meant that the number of moles of basic compound used is equal to the number of the carboxyl functions present in hyaluronic acid. The possibility of varying the ratio between moles of acid and moles of base enables modulation of the characteristics of the products that can be obtained according to the properties desired.

The derivatives between hyaluronic acid and purine/pyrimidine bases according to the present invention may react also with other compounds that will be able to form bonds of an ionic type with the possible residual carboxyl groups present on hyaluronic acid. For example, there may be used, for further salification of derivatives of hyaluronic acid, modified amino acids, polyamino acids, growth factors, active principles with pharmacological activity, angiogenesis inhibitor factors, etc.

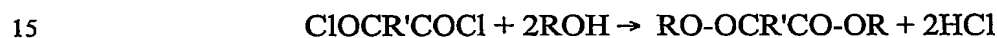
In each of the cases described above, the possibility of rapidly hydrolysing the ionic bond between the aforesaid compounds and hyaluronic acid, which is in turn salified with at least one purine/pyrimidine base, makes the product immediately available just as it is, but enables its vehicling and delivery to the "target" identified with equal rapidity, thanks to the considerable ease of diffusion of the derivatives of hyaluronic acid according to the invention.

The reaction of salification between hyaluronic acid and purine/pyrimidine base may occur by means of reaction between an acid and a free base, or else between an acid in the form of salt (carboxylate) and a base also salified with organic and/or inorganic acids. In this latter case, the reaction envisages an ion exchange so as to obtain the desired product in appropriate conditions. The reaction may be conducted using stoichiometric amounts of reagents or an excess of one of the two components, in which case the salt and a co-ordination of the reagent in excess is obtained by means of the formation of hydrogen bonds. In this way it is possible to modulate the characteristics of the hyaluronate derivatives (hyaluronic acid and purine/pyrimidine base) and possibly of further active principles or other components associated thereto, as described above. Said active principles or other components

may be associated to the hyaluronate derivatives by co-ordination by means of the formation of hydrogen bonds. To the product obtained it is possible to add both organic and inorganic acids or bases (for example, glucosamine and glucuronic acid), with the purpose of modifying its pH and possibly its pharmacological activity, if present.

Yet again according to the present invention, hyaluronic acid may be cross-linked by means of formation of bonds of a covalent type (breaking of a preceding bond of a covalent type and formation of a new bond once again of a covalent type) between side chains. In particular, in the cross-linking reaction there may be involved both hydroxyl groups (OH) and carboxyl groups (COOH) present on the molecule of hyaluronic acid.

For example, in the case of the cross-linking reaction by means of esterification of the hydroxyl groups, this takes place rapidly even at room temperature using chlorides of organic acids according to the following scheme:



where ROH represents hyaluronic acid. The reaction may, for example, be conducted in an inert solvent such as chlorobenzole or in a solvent such as DMF, which neutralises the hydrochloric acid produced by the reaction. The reaction may be performed on free hyaluronic acid or on its inorganic salt or organic salt thereof.

Likewise, the cross-linking reaction by means of esterification of the carboxyl groups, envisages for example the use of chloro-organic compounds, which react with the carboxyl groups of hyaluronic acid according to the following scheme:



Where RCOOH represents hyaluronic acid. The reaction may be performed on free hyaluronic acid, an inorganic salt thereof (in this case, instead of HCl, the corresponding hydrochloride salt is obtained) or an organic salt.

#### EXAMPLE 5 - CROSS-LINKING BY ESTERIFICATION WITH PHOSGENE

An aqueous solution of sodium hyaluronate (1 g in 500 ml of water) is prepared.

There is then prepared a solution of  $\text{COCl}_2$  (phosgene) 0.01 g - 0.005 g in 20-50 ml of chloroform. The amount of phosgene given is simply indicative and may vary according to the degree of cross-linking desired. The solution of chloroform is added to that of sodium hyaluronate under stirring, thus obtaining the cross-linking.

- 5 If the reaction is conducted in the absence of stirring, keeping the two phases (water and chloroform) in contact, in the interface there is formed a film of cross-linked polymer that may be removed continuously. The reaction takes place rapidly at room temperature.

- According to a preferred aspect of the present invention, the derivatives of
- 10 hyaluronic acid according to the invention are used as active principles in the preparation of medicaments. They are suitable for being formulated with appropriate excipients, for example those generally used in the cosmetic field, such as beeswax, jojoba oil, isostearyl isostearate, fatty acids, triglycerides, propylene glycol, laurilic alcohol and hydroxypropyl methylcellulose. There may moreover be added to these
- 15 stabilizing active principles, antioxidants and preservatives, either alone or in combination with one another. As stabilizing agents butylhydroxyanisole, citric acid, tocopherol, or sodium thiophosphate may be used. As preservatives, esters of p-hydroxybenzoic acid or imidazolidinurea may be used.

## CLAIMS

1. A derivative between hyaluronic acid and at least one heterocyclic compound derived from purine and/or from pyrimidine, said derivative being provided with at least one bond of a ionic type between said acid and said at least one heterocyclic compound.  
5
2. The derivative according to Claim 1, characterized in that said hyaluronic acid is hyaluronic acid of high molecular weight.
3. The derivative according to Claim 2, characterized in that said hyaluronic acid has a molecular weight of between 400 000 and 4 million dalton.
- 10 4. The derivative according to Claim 3, characterized in that said hyaluronic acid has a molecular weight of between 800 000 and 3.5 million dalton.
5. The derivative according to Claim 4, characterized in that said hyaluronic acid has a molecular weight of between 1.5 and 3 million dalton.
6. The derivative according to Claim 1, characterized in that said hyaluronic acid is hyaluronic acid of low molecular weight.  
15
7. The derivative according to Claim 1, characterized in that said heterocyclic compound is chosen between: adenine, guanine, thymine, cytosine, uracyl, 5,6 dihydrouracyl, 1-methyluracyl, 3-methyluracyl, 5-hydroxymethyluracyl, 2-thiouracyl, N<sup>4</sup>-acetylcytosine, 3-methylcytosine, 5-methylcytosine, 5-hydroxymethylcytosine, 1-methyladenine, 2-methyladenine, 7-methyladenine, N<sup>6</sup>-methyladenine, N<sup>6</sup>, N<sup>6</sup>-dimethyladenine, N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine, 1-methylguanine, 7-methylguanine, N<sup>2</sup>-methylguanine, and N<sup>2</sup>, N<sup>2</sup>-dimethylguanine.  
20
8. The derivative according to Claim 1, characterized in that said heterocyclic compound is chosen from between adenine, guanine, thymine, cytosine.  
25
9. The derivative according to Claims 1 and 7, characterized in that said bond of a ionic type is obtained between said acid and at least two of said heterocyclic compounds that are the same as or different from one another.
10. The derivative according to Claims 1 and 7, characterized in that it is guanine

hyaluronate.

11. The derivative according to Claims 1 and 7, characterized in that it is adenine hyaluronate.
12. The derivative according to Claim 1, characterized in that it is associated to at  
5 least one different organic compound.
13. The derivative according to Claim 12, characterized in that said organic compound is chosen from between natural amino acids, their oligomers and polymers (peptides).
14. The derivative according to Claim 13, characterized in that it is guanine  
10 hyaluronate, polylysine.
15. The derivative according to Claim 13, characterized in that it is adenine hyaluronate, polylysine.
16. The derivative according to Claim 1, characterized in that it is cross-linked.
17. The derivative according to Claim 16, characterized in that said cross-linking  
15 involves at least one hydroxyl group and/or at least one carboxyl group present on said hyaluronic acid.
18. The derivative according to Claim 16, characterized in that said cross-linking is obtained with phosgene.
19. A process for the preparation of a derivative between hyaluronic acid and at  
20 least one heterocyclic compound according to Claim 1, characterized in that hyaluronic acid or a salt thereof is set to react with at least one heterocyclic compound in free or salified form.
20. The process for the preparation of a derivative according to Claim 12,  
characterized in that said derivative of hyaluronic acid or a salt thereof is set to  
25 react with at least one organic compound in free or salified form.
21. Use of a derivative according to Claim 1 in the cosmetic field.
22. Use of a derivative according to Claim 1 in the pharmaceutical field.
23. Cosmetic or pharmaceutical compositions comprising the compounds referred to in Claim 1.

24. Cosmetic or pharmaceutical compositions comprising the compounds referred to in Claim 12.
25. Use of the compositions referred to in Claims 23 and 24 in the cosmetic and/or pharmaceutical field.

5

## INTERNATIONAL SEARCH REPORT

PCT/03/02946

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C08B37/08 C08L5/08 A61K47/36 A61K31/728 A61K7/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C08B C08L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 555 898 A (FIDIA SPA) 18 August 1993 (1993-08-18) page 6, line 13 - line 24 page 3, line 58 page 4, line 21 claims 1-3,7	1-6, 9, 19, 21-25
A	WO 00 01733 A (BELLINI DAVIDE ; FIDIA ADVANCED BIOPOLYMERS SRL (IT); TOPAI ALESSAN) 13 January 2000 (2000-01-13) page 2, line 17 - line 28 page 5, line 1 - line 3 page 5, line 30 - page 6, line 7 page 6, line 19 - line 22 page 7, line 3 - line 6 page 9, line 27 - line 31	1-6, 19, 21-25

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

14 November 2003

Date of mailing of the international search report

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